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Genetic polymorphisms in catalase and CYP1B1 determine DNA adduct formation by benzo(a)pyrene *ex vivo*

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Genetic polymorphisms can partially explain the large inter-individual variation in DNA adduct levels following exposure to polycyclic aromatic hydrocarbons. Effects of genetic polymorphisms on DNA adduct formation are difficult to assess in human studies because exposure misclassification attenuates underlying relationships. Conversely, *ex vivo* studies offer the advantage of controlled exposure settings, allowing the possibility to better elucidate genotype–phenotype relationships and gene–gene interactions. Therefore, we exposed lymphocytes of 168 non-smoking volunteers *ex vivo* to the environmental pollutant benzo(a)pyrene (BaP) and BaP-related DNA adducts were quantified. Thirty-four genetic polymorphisms were assessed in genes involved in carcinogen metabolism, oxidative stress and DNA repair. Polymorphisms in *catalase* (*CAT*, rs1001179) and cytochrome P450 1B1 (*CYP1B1*, rs1800440) were significantly associated with DNA adduct levels, especially when combined. Moreover, reverse transcription–polymerase chain reaction (RT–PCR) analysis in a subset of 30 subjects revealed that expression of catalase correlated strongly with expression of CYP1B1 ($R = 0.92$, $P < 0.001$). To further investigate the mechanism by which catalase influences CYP1B1 and how they simultaneously affect BaP-related DNA adduct levels, catalase expression was transiently knocked down in the human lung epithelial cell line A549. Although catalase knockdown did not immediately change CYP1B1 gene expression, recovery of catalase expression 8 h after the knockdown coincided with a 2.2-fold increased expression of CYP1B1 ($P < 0.05$). We conclude that the genetic polymorphism in the promoter region of *CAT* may determine the amount and activity of catalase, which may subsequently regulate the expression of CYP1B1. As a result, both genetic polymorphisms modulate DNA adduct levels in lymphocytes by BaP *ex vivo*.

Introduction

Genetic stability is challenged by many factors, including exposure to genotoxic chemical compounds such as polycyclic aromatic hydrocarbons (PAHs). PAHs such as benzo(a)pyrene (BaP) are widely distributed environmental contaminants that are able to upregulate a multitude of genes, including the cytochrome P450 isoforms CYP1A1 and CYP1B1. These enzymes metabolise BaP to more soluble and excretable products but at the same time activate certain metabolites into the highly reactive form BaP-7,8-dihydrodiol-9,10-epoxide (BPDE) that is able to bind to DNA to form bulky DNA adducts. When unrepaired, DNA adducts can lead to mutations that initiate carcinogenesis. Therefore, bulky DNA adducts are markers for both the exposure to genotoxic aromatic compounds and the ability of the individual to metabolically activate carcinogens and to repair DNA damage (1). Nevertheless, individuals with similar exposures have highly different DNA adduct levels (2), which may in part be explained by genetic polymorphisms in genes involved in the process of DNA adduct formation and repair. These variations have been studied extensively and multiple genetic polymorphisms affecting BPDE-DNA adducts have been identified, including genes involved in metabolism (CYP1A1, GSTM1, GSTT1 (3–5)), DNA repair (ERCC1, XPD (6)) and oxidative stress (7).

We previously showed that single genetic polymorphisms are unable to adequately account for the inter-individual variation but rather by combinations of relevant genetic polymorphisms and the level of exposure (8). The level of exposure is often insufficiently characterised in large epidemiological studies that investigate the impact of genetic variation on DNA damage or disease, leading to misclassification and attenuation of underlying relationships. On the other hand, exposure can be well controlled in *in vitro* or *ex vivo* studies, offering the possibility to elucidate genotype–phenotype relationships and gene–gene interactions more reliably. Interestingly, the level of BPDE-DNA adduct formation *ex vivo* in lymphocytes was related to lung cancer risks (9), further offering support for using *ex vivo* experiments to investigate sources of inter-individual variation in cancer susceptibility. Therefore, lymphocytes of healthy volunteers were incubated with 1 µM BaP for 18 h. We assessed 34 single nucleotide polymorphism (SNPs) based on a priori knowledge (10) and determined which SNPs were responsible for differences in BaP-related DNA adduct levels. One SNP in catalase (*CAT,* rs1001179) and one in *CYP1B1* (rs1800440) had a significant impact on BaP-related DNA adducts formation and further experiments were performed to study genotype–phenotype relationships and gene–gene interactions in lung epithelial cells (A549) in which catalase was transiently knocked down by siRNA.

Materials and methods

Study population

The previously described study population consisted of 114 females and 54 males, aged 18–45 with an average of 29 and 28 years for males and females, respectively (10). These healthy volunteers were recruited through advertisements in local newspapers and were non-smokers and did not use medication (except for oral contraceptives by all female volunteers) or vitamin supplementation. Subjects were fully informed about the details of the study and gave their written informed consent. The Medical Ethical Committee of Maastricht University and the Academic Hospital Maastricht approved the protocol.

Collection and treatment of samples

Venous blood samples were collected between 8 and 9 AM in 10 ml vacuum lithium heparin tubes (venoject II, Terumo-Europe, Leuren, Belgium). For isolation of lymphocytes, heparinised blood was 1:1 diluted with phosphate-buffered saline (PBS, pH 7.4) and layered on Lymphoprep™ (Axis-shield, Oslo, Norway) in a leucosep tube (Greiner Bio-one, Frickenhausen, Germany). Lymphocytes were separated by 20 min 860*g* centrifugation at room temperature and subsequently washed and treated with 25 µM hydrogen peroxide for 1 h at 37°C or 1 µM BaP for 18 h at 37°C as described by Wilms *et al*. (10)

Comet assay

Following incubation with hydrogen peroxide, cell suspensions were diluted 1:4 in 0.5% low melting-point agarose and added to microscope slides precoated with a layer of 1.5% normal melting-point agarose and put at 4°C for 45 min. Cells were lysed (0.25 M NaOH, 0.1 M EDTA, 0.01 M Tris, 2.5 M NaCl, 1% Triton X-100 and 10% DMSO, adjusted to pH 10) for 1 h at 4°C, washed with PBS, placed in electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH 13) for 40 min for denaturation and subsequently separated by electrophoresis for 30 min at 25 V and 300 mA. The slides were washed twice with PBS for 10 min and stained with ethidium bromide (10 µg/ml) and visualised using a Zeiss Axioskop fluorescence microscope. About 50 randomly selected nuclei were analysed per slide using the Comet assay III software program (Perceptive Instruments, Havervill, UK).

Genotyping

DNA was isolated from lymphocytes by standard phenol extraction procedures. The Cancer SNP 500 database was used to obtain DNA sequences and allele frequencies (http://snp500cancer.nci.nih.gov) of 34 SNPs (Supplementary Table I, available at *Mutagenesis* Online). SNPs were analysed using a multiplex PCR method developed by Knaapen *et al.* (11) and further refined by Ketelslegers *et al.* (8). Genotyping of 10% of the samples was performed in duplicate to ensure reliability.

^{32}P post-labeling of BaP-related DNA adducts

DNA adduct levels were determined according to the nuclease P1 enrichment technique originally reported by Reddy and Randerath (12) with the modifications described by Godschalk *et al*. (13). Three standards with known BaP-related DNA adduct levels were analysed in parallel for quantification purposes. Adduct spots that chromatographed at the same position as the BaP-related DNA adduct standards were considered to be derived from BaP and were quantified using Phosphor-Imaging technology (Fujifilm FLA-3000, Rotterdam, The Netherlands).

Cell culture and siRNA transfection

The human epithelial lung carcinoma cell line A549 obtained from the American Tissue Culture Collection (ATCC, Rockville, MD, USA) were cultured in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS, Invitrogen, Breda, The Netherlands) and 1% penicillin/streptomycin (Sigma), and maintained at 37°C in a 5% CO_2 atmosphere. Cells were seeded at 10% confluence 1 day before treatment in antibiotic free medium and maintained at 37°C in a 5% CO_2 atmosphere. Cells were transfected with 10 nM siGENOME SMARTpool siRNA specific for human catalase (Dharmacon, Lafayette, CO, USA) using DharmaFECT (Dharmacon). The transfections were performed according to manufacturer's instructions. After 24 h, the medium was removed and replaced with regular culture medium for a further 8 h.

Real-time quantitative PCR

RNA for real-time quantitative PCR was isolated from lymphocytes or A549 cells by standard phenol extraction procedures. The RNA samples were spectrophotometrically quantified using Nanodrop 1000 (Thermo Scientific, Waltham, MA, USA). Complementary DNA (cDNA) synthesis was performed using the iScript cDNA Synthesis kit (Biorad, Veenendaal, The Netherlands) starting with 1 µg of RNA. The generated cDNA was diluted 25× in RNase free water. Real-time PCR was performed using the MyiQ Single Color RT–PCR detection system (Biorad) and Sensimix Sybr Green (Quantace, London, UK). 5 µl diluted cDNA and 0.3 µM primers (Supplementary Table II, available at *Mutagenesis* Online) in a total volume of 25 µl were used. Samples were amplified under the following conditions: 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 45 s. The PCR was checked for non-specific products by performing a melting curve analysis (65°C–95°C). Data were analysed using the MyiQ Software system (Biorad) and were expressed as relative gene expression (fold increase) using the $2^{-\Delta\Delta Ct}$ method. The stably expressed gene β-actin was included as reference.

Statistical analysis

Results are expressed as mean ± standard error of the mean. SPSS version 16 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. BaP-related DNA adducts were not normally distributed and were therefore log transformed before statistical analysis. To examine differences between the three groups of polymorphic genotypes (i.e. homozygous wild type, heterozygotes and homozygous variant), a one-way analysis of variance test with Bonferroni post hoc multiple comparison correction was used. Linear regression was used to assess relations. The two-way analysis of variance test with Bonferroni post hoc multiple comparison correction was used to examine the effect of catalase knockdown. Differences were considered to be statistically significant when $P < 0.05$.

Results

SNPs in CYP1B1 and catalase are linked to ex vivo BaP-related DNA adduct formation

Of the 34 SNPs, we obtained (Supplementary Table I, available at *Mutagenesis* Online); only two were related to BaP-related DNA adduct levels *ex vivo*. These were the polymorphisms in *CYP1B1* (rs1800440) and *catalase* (rs1001179). Individuals carrying one or both variant allele of *CYP1B1*-N453S showed a statistically significant lower amount of BaP-related DNA adducts compared with individuals that were homozygous for the wild-type allele (Figure 1A). Individuals carrying one or both variant alleles of *CAT* C-262T had higher levels of BaP-related DNA adducts compared with individuals that were homozygous for the wild-type allele (Figure 1B). Lowest

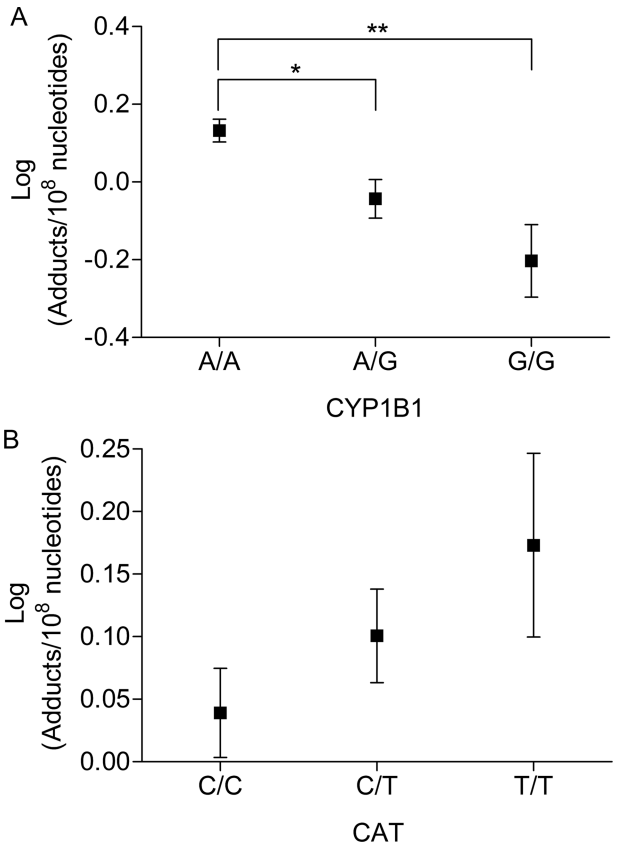

Fig. 1. Modulation of *ex vivo* induced BaP-related DNA adducts by SNPs in *catalase* and *CYP1B1*. Lymphocytes of 168 volunteers were incubated with 1 µM BaP for 18 h and DNA adducts were measured using ^{32}P post-labeling. The effect of the (**A**) *CYP1B1*-N453S polymorphism and the (**B**) *CAT*-262C/T polymorphism on BaP-related DNA adduct formation was determined. DNA adduct data were log transformed and presented as mean ± standard error of the mean ($n = 168$, $^{*}P < 0.05$, $^{**}P < 0.01$).

BaP-related DNA adduct levels were observed in subjects that carried the combined homozygous *CYP1B1* variant alleles and the homozygous wild-type alleles for *CAT*.

Phenotypic effect of SNP in promoter region of CAT

As the SNP in catalase is located in the promoter region, we sought to determine whether gene expression would be affected. Therefore, mRNA levels were determined (Figure 2) in unexposed peripheral blood lymphocytes of 30 subjects selected on basis of their genotype (10 of each genotype). Individuals homozygous for the wild-type allele of *CAT* (CC) had ~1.9-fold lower catalase mRNA levels than individuals that were homozygous for the variant allele ($P < 0.001$). To assess whether increased expression of CAT also resulted in a concomitant increased catalase activity, the ability to prevent single-strand breaks (SSB) induced by H_2O_2 was determined (Figure 3). To this end, lymphocytes were incubated with H_2O_2 and a comet assay was performed to measure SSB. Samples from individuals that were homozygous for the wild-type allele of *CAT* (CC) had ~1.5-fold higher amount of SSB than samples from subjects carrying at least one T-allele ($P < 0.001$). This is consistent with the lower mRNA expression levels observed in lymphocytes of subjects carrying at least one T allele.

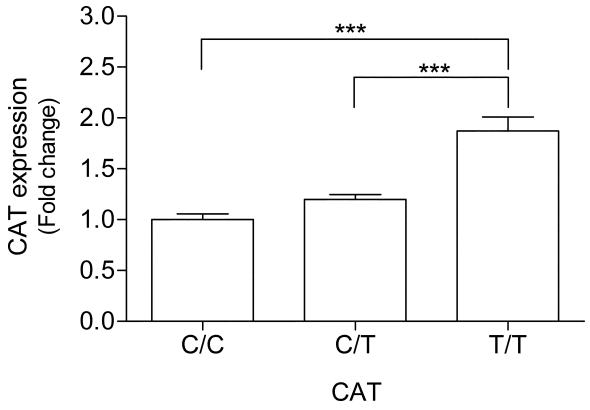

Fig. 2. SNP in promoter region of *catalase* affects CAT expression. The effect of the *CAT*-262C/T polymorphism on catalase gene expression was determined in unexposed lymphocytes. Data are presented as fold increase compared with the C/C genotype ($n = 30$, 10 in each group, ***$P < 0.001$).

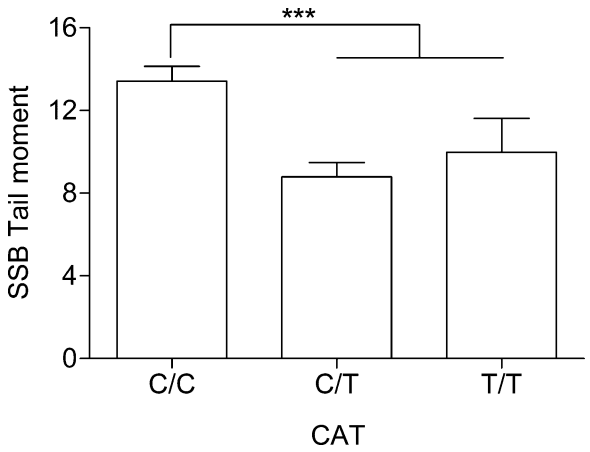

Fig. 3. *CAT* polymorphism reduces H_2O_2-induced SSBs. The effect of *CAT*-262C/T polymorphism on catalase phenotype was determined in H_2O_2-exposed lymphocytes. Lymphocytes were incubated with 25 µM H_2O_2 for 1 h and SSBs were measured using the comet assay. Data are presented as mean ± standard error of the mean ($n = 135$, ***$P < 0.001$).

Expression of catalase strongly correlated with expression of CYP1B1

To further define how catalase expression effects BaP-related DNA adduct levels, catalase mRNA levels were correlated with the expression of CYP1B1 and a strong significant correlation was observed ($P < 0.001$, $R = 0.92$, Figure 4). The genetic polymorphism in *CAT*, which regulates the level of gene expression of CAT, thus seems to co-regulate the expression of CYP1B1 in human lymphocytes. Indeed, the expression of CYP1B1 was approximately 2-fold higher in carriers of the *CAT* TT-genotype than in the CC-genotype subjects but this did not reach statistical significance ($P = 0.08$). No difference was observed between CYP1B1 mRNA expression of male and female volunteers (data not shown).

In vitro modulation of catalase expression alters CYP1B1 mRNA levels

To further investigate the mechanism by which catalase affects CYP1B1 gene expression, A549 lung epithelial cells lines were used to investigate the effect of changing catalase gene expression on CYP1B1. A statistically significant correlation in the expression of catalase and CYP1B1 was observed in unexposed cells ($P < 0.001$, $R = 0.96$, Figure 4). From the *ex vivo* observation, we hypothesised that knocking down the expression of catalase would also downregulate the expression of CYP1B1. After knockdown of catalase expression, only 15% of the catalase mRNA remained following 24 h of siRNA treatment (Figure 5). The expected simultaneous downregulation of CYP1B1 gene expression was not observed. On the other hand, the removal of the siRNA resulted in the recovery of CAT expression, which coincided with a 2.2-fold upregulation of CYP1B1 expression ($P < 0.001$).

Downregulation of catalase is expected to increase intracellular oxidative stress; the resulting activation of the nuclear erythroid 2-related factor 2 (Nrf2) pathway may be a potential

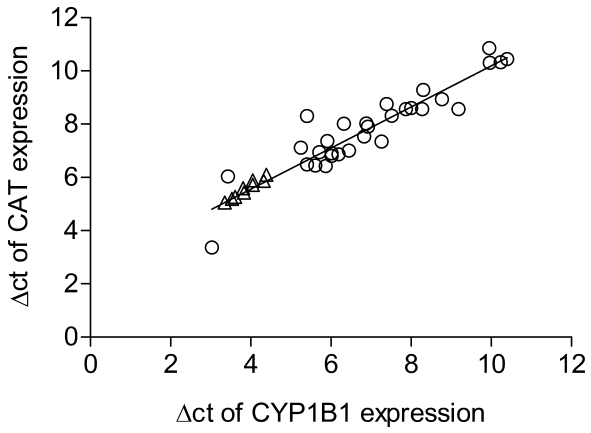

Fig. 4. Expression of catalase correlates strongly with expression of CYP1B1. mRNA levels of catalase and CYP1B1 were measured in unexposed lymphocytes, $n = 30$ (circle), and A549 cells, $n = 9$ (triangle). Data are presented as Ct value difference between CAT and the housekeeping gene. Regression line shown belongs to gene expression of lymphocytes but also fits the results in A549 cells.

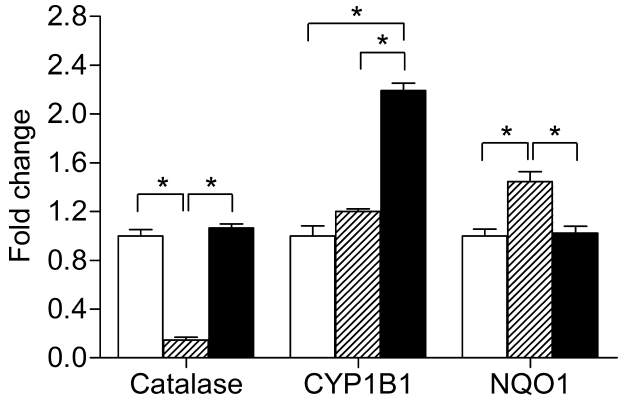

Fig. 5. CYP1B1 mRNA levels are increased upon recovery of catalase mRNA levels 8 h after a transient knockdown. mRNA levels of catalase, CYP1B1 and NQO1 in A549 were measured after no treatment (white), 24 h of catalase siRNA (striped), 24 h of catalase siRNA followed by 8 h recovery without siRNA (black). Data are presented as mean ± standard error of the mean fold increase compared with no treatment ($n = 3$ for each bar, $^*P < 0.001$).

explanation for the initial lack of response in CYP1B1 expression since CYP1B1 expression is also known to be modulated by Nrf2 (14). Therefore, gene expression of the reporter gene NAD(P)H dehydrogenase quinone 1 (NQO1), which is regulated via the Nrf2 pathway, was determined and interestingly a significant upregulation of NQO1 (~1.4-fold) was observed during catalase knockdown. This suggests that an activation of the Nrf2 pathway prevents CYP1B1 to be downregulated in parallel with catalase after its knockdown. On the other hand, the subsequent removal of the siRNA and resultant restoration of catalase expression coincided with an upregulation of CYP1B1 expression. These *in vitro* studies confirm that catalase may affect CYP1B1 gene expression.

Discussion

PAHs such as BaP are carcinogens that humans are unknowingly and/or unavoidably exposed to on a daily basis. The ability of an individual to cope with these carcinogens may determine the risk of developing cancer. This intrinsic risk varies within the population and genetic polymorphisms in genes such as those involved in BaP metabolism may be a source of this disparity. Previous studies showed that certain genetic polymorphisms are related to an increase in adduct formation (3,15). However, phenotypic effects of certain genetic polymorphisms are difficult to assess in epidemiological studies because exposure misclassification attenuates underlying relationships. Therefore, we exposed cells to a single compound to further elucidate genotype–phenotype relationships and report that genetic polymorphisms in *catalase* and *CYP1B1* interact in determining the levels of BaP-related DNA adducts *ex vivo*.

Of the 34 polymorphisms investigated, only two resulted in a difference in BaP-related DNA adduct formation. The *CYP1B1*-N453S polymorphism has previously been linked to altered cancer risks and aggressiveness of hormone-dependent cancers (16,17). This could be explained by the increased degradation rate of CYP1B1 and subsequent decreased CYP1B1 activity observed in individuals with the 453S allele (18). Decreased CYP1B1 activity can result in a decreased genotoxicity from estrogen metabolism (19). Interestingly, estrogen metabolism shows large overlap with the metabolism of PAH, such as BaP. CYP1B1 is also capable of metabolising BaP to its reactive metabolites. This metabolic activation is usually ascribed to CYP1A1 but the expression of CYP1A1 is minimal in lymphocytes. In our sample population, expression of CYP1A1 was more than 18-fold lower than the expression of CYP1B1 (data not shown). The *CYP1B1*-N453S variant alleles appear to be protective since individuals carrying one or two variant alleles had lower levels of BaP-related DNA adducts after *ex vivo* exposure to BaP (Figure 1).

The other polymorphism that influenced BaP-related DNA adduct formation in our population was catalase C-262T (rs1001179). Catalase reduces oxidative stress by catalysing the conversion of H_2O_2 into water and oxygen. The location of this polymorphism in the promoter suggests that it is involved in the regulation of catalase gene expression. Therefore, we measured catalase mRNA levels in lymphocytes from individuals carrying different alleles. The subjects carrying the variant allele had higher expression of CAT compared with subjects carrying the wild-type allele. This finding is in concordance with Forsberg *et al.* (20) who showed that the C/T polymorphism in the promoter region of the *CAT* gene induced the transcriptional activity of the promoter. The induced catalase gene expression also coincide with the induced activity of catalase in subjects carrying at least one T-allele since they had lower levels of SSB induced by H_2O_2.

The observation that the expression of CYP1B1 was approximately 2-fold higher in carriers of the *CAT* TT-genotype than in the CC-genotype subjects and because a strong correlation was observed between the expression of CAT and the expression of CYP1B1 led to the hypothesis that the genetic polymorphism in *CAT* would affect DNA adduct formation induced by BaP *via* its effect on CYP1B1 expression. Previous studies in hepatoma cells have shown that oxidative stress decreased the expression of CYP1A1, which like CYP1B1 is a member of the CYP1 family (21–23). This oxidative stress–mediated repression was mediated by a nuclear factor 1 (NF1) binding to the promoter region of these CYPs (22). The expression of CYP1A1 and CYP1B1 is activated by binding of a ligand, such as BaP, to the aryl hydrocarbon receptor (AhR). Upon ligand binding, AhR translocates into the nucleus and dimerises with the AhR nuclear translocator (ARNT), which can bind to the TNGCGTG consensus sequence in the xenobiotic-responsive elements of target genes (24). This AhR-ARNT complex synergises with other transcription factors including NF1, thereby activating gene expression, suggesting that high levels of H_2O_2 could also result in reduced transcription of CYP1B1. We found a strong correlation between catalase expression and CYP1B1 expression ($R > 0.92$) in lymphocytes and A549 cells. Thus, we hypothesised that the SNP in catalase determines the amount of CYP1B1 and the SNP in CYP1B1 subsequently determines the activity of the enzyme.

To further study the combined effect of *CAT* and *CYP1B1* polymorphisms on BaP-related DNA adduct formation, we transiently knocked down catalase by siRNA. Indeed, the recovery of CAT expression after removal of the siRNA coincided with increasing levels of CYP1B1 mRNA levels. However, the effective knockdown of catalase expression to less than 15% of the original level did not result in the expected decrease of CYP1B1 gene expression. This suggests that under circumstances of oxidative stress, other mechanisms are capable of keeping CYP1B1 gene expression intact. A likely candidate was the Nrf2 that when activated

significantly induced AhR, CYP1A1 and CYP1B1 transcriptions in mouse embryonic fibroblasts (14). In response to oxidative stress, Nrf2 is activated and upregulates antioxidant and detoxifying genes, including NQO1 (25). In our study, the increase in NQO1 mRNA levels suggests that the knockdown of catalase is indeed related to increased oxidative stress levels and activation of Nrf2. The opposing effects of both NF1 and Nrf2 eventually resulted in an initial unchanged CYP1B1 gene expression. However, when catalase mRNA levels started to increase after removal of the siRNA, oxidative stress quickly decreased and the Nrf2 was deactivated (since NQO1 expression went back to baseline levels). We speculate that as a result of the restored expression of catalase, CYP1B1 expression further increased and was over-compensated (>2-fold increased expression). These *in vitro* studies confirm that catalase affects CYP1B1 gene expression.

We propose a model in which the genetic polymorphism in the promotor region of *CAT*-262C→T determines the amount of intracellular oxidative stress, which co-regulates the expression of CYP1B1 by reducing the amount of H_2O_2 present in the cells. Oxidative stress would inactivate or activate relevant transcription factors (NF1 and Nrf2). The genetic polymorphisms in *CYP1B1* subsequently modulates BaP metabolism *ex vivo* in lymphocytes. Thus, the SNP in *CAT* determines the amount of CYP1B1, whereas the SNP in *CYP1B1* determines its activity. These *ex vivo* data indicate that inter-individual variations in DNA adduct levels depend on gene–gene interactions, which need further attention for application in *in vivo* studies. Understanding gene–gene interactions allows the identification of relevant combinations of SNPs to assess an individual's cancer susceptibility.

Supplementary data

Supplementary Tables I and II are available at *Mutagenesis* Online.

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Conflicts of interest: none declared.

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